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Contaminants in Commercial Preparations of ‘Purified’ Small Leucine-Rich Proteoglycans May Distort Mechanistic Studies

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ABSTRACT

This paper reports the perplexing results that came about because of seriously impure commercially available reagents. Commercial reagents and chemicals are routinely ordered by scientists and are expected to have been rigorously assessed for their purity. Unfortunately, we found this assumption to be risky. Extensive work was carried out within our laboratory using commercially-sourced preparations of the small leucine-rich proteoglycans, decorin and biglycan, to investigate their influence on nerve cell growth. Unusual results compelled us to analyse the composition and purity of both preparations of these proteoglycans using both mass spectrometry and Western blotting, with and without various enzymatic deglycosylations. Commercial ‘decorin’ and ‘biglycan’ were found to contain a mixture of proteoglycans including not only both decorin and biglycan but also fibromodulin and aggrecan. The unexpected effects of ‘decorin’ and ‘biglycan’ on nerve cell growth could be explained by these impurities. Decorin and biglycan contain either chondroitin or dermatan sulphate glycosaminoglycan chains whilst fibromodulin only contains keratan sulphate and the large (>2,500 kDa), highly glycosylated aggrecan, contains both keratan and chondroitin sulphate. The different structure, molecular weights and composition of these impurities significantly affected our work and any conclusions that could be made. These findings beg the question as to whether scientists need to verify the purity of each commercially obtained reagent used in their experiments. The implications of these findings are vast, since the effects of these impurities may already have led to inaccurate conclusions and reports in the literature with concomitant loss of researchers’ funds and time.

Short title: *Impurities in commercial preparations of decorin and biglycan*

Keywords: Commercial preparations, small leucine-rich proteoglycans (SLRPs), decorin, biglycan, mass spectrometry, , glycosaminoglycan

Abbreviations: CS, chondroitin sulphate; DRG, dorsal root ganglion; DS, dermatan sulphate; ECM, extracellular matrix; GAG, glycosaminoglycan; KS, keratan sulphate; PG, proteoglycan; SLRP, small leucine-rich proteoglycan.

INTRODUCTION

For several decades it has been known that extracellular matrix (ECM) molecules can provide topographical cues and guidance for neuronal growth. Proteoglycans (PGs) are a major component of the ECM in several tissues in our body and have been shown to influence nerve growth in many systems from the developing central nervous system (1,2) to the intervertebral disc (3-5). For example, they are reported to inhibit outgrowth of sensory neurons (6), reduce astrogliosis or, indirectly, promote axon growth of adult sensory neurons (7), with chondroitin sulphate-PGs acting through receptor protein tyrosine phosphatase sigma (8). PGs are diverse molecules which can range in molecular weight from 25 kDa to 3 million kDa, depending on the length of their protein core and the number and type of glycosaminoglycan (GAG) chains attached (which can vary from 1 to 150). In addition, PGs may occur as a single molecule or, as in the case of aggrecan or versican, be linked to hyaluronan to form very large aggregates. The exact attribute of the PG which affects nerve growth is not clear as it may be the core protein, GAG side chains, GAG sulphation, the degree of aggregation in the case of aggrecan, or a combination of these factors.

One of the main areas of interest within our research group is the biology and degeneration and regeneration of the intervertebral disc. Degeneration of the disc is a common phenomenon, which is of interest clinically because of its association with one of the largest health burdens in the Western world, that of back pain (9-12). Previously, a large twin study found degeneration of the disc in the lumbar spine to be the single most important risk factor for low back (13). The intervertebral disc is a connective tissue which is unusual in having little vascularisation or innervation. In the healthy disc, nerves are restricted to the outer few millimetres of the disc; in the lumbar region, responsible for most pathology, this is typically between 4 and 5 mm. When discs become degenerate the effects can be widespread resulting in abnormal loads being transmitted to the adjacent tissue and structures (14) and is typified by microscopic and biochemical changes, with altered cellular metabolism and structural matrix derangements (15). The loss of PG and water from the matrix leads to cracks and fissures forming within the disc and its adjacent endplates, and these are often accompanied by neural and vascular ingrowth (16-19).

PGs, with their considerable hydrophilic capacity, are fundamental to the main function of the disc, that of weight bearing in the spine and it is these molecules that are the first to alter when discs degenerate (20). Aggrecan is the main PG in the disc and it becomes enzymatically fragmented during age-related degeneration, both in terms of the protein core being cleaved and the GAG chains becoming shorter, resulting in the reduced PG and GAG content in degenerative discs (21). In previous studies, our group have demonstrated that disc aggrecan inhibits the growth of neurites in a dose dependent manner (3), hence providing a mechanism and linkage for the loss of PG and increased innervation in degenerate discs.

Whilst PGs make up 13-61% of the dry weight in the disc (22) and the majority of this is aggrecan, there are other PG family members present in the matrix, albeit at a lower concentration, such as the small leucine-rich proteoglycans (SLRPs). Several SLRPs are known to occur in human discs and include biglycan, decorin, fibromodulin, keratocan and lumican (23-26). In addition, some of these SLRPs have been shown to be increasingly fragmented with disc degeneration in humans (23), as well as in the discs of chondrodystrophoid breeds of dog, which are prone to disc disorders (27). In addition to aggrecan, decorin and biglycan in particular have been shown to influence nerve growth and function. For example, decorin promotes both axon growth in spinal cord injury in the rat (7) and influences scar formation at the site of nerve injury (28) which in turn impacts on nerve growth whilst biglycan contributes to synapse stability (29). Decorin together with TGF β (which binds to decorin and modulates its effect) may have a biphasic expression in nerve injury, likely to be part of a nerve repair mechanism (30).

This study highlights the importance of evaluating the composition of commercially available preparations. We had set out to determine if decorin and biglycan might influence nerve growth in model systems used previously in our laboratory to study the influence of aggrecan (3,31,32), but unexpected results led us to investigate the composition of both commercially purchased SLRP molecules. This in turn has led us to be increasingly sceptical about the purity of some commercially available biochemicals.

EXPERIMENTAL PROCEDURES

Culture and assessment of neuronal growth

Dorsal root ganglia (DRGs) were obtained as previously described and used as a source of sensory neurons (3). Briefly, DRGs were dissected from white leghorn chickens at day 10 of embryogenesis, cut into quarters and then at least 5 explants were seeded onto a Petri dish coated with various substrates. These included the neural-permissive type I collagen and either 'decorin' (D8428, Sigma, Lot numbers 101M4033V, 029k4058) or 'biglycan' (B8041, Sigma, Lot number 070M4068). The substrate coatings were prepared by flooding the wells of a 12-well tissue culture plate with a solution of nitrocellulose in methanol (0.4cm²/ml) which was then allowed to dry. Strips of Whatman's filter paper, soaked in solutions of 'decorin' ranging from 10-1,000 µg/ml or 'biglycan' ranging from 2-500 µg/ml diluted in phosphate buffered saline (PBS) with 10% rhodamine-B, were blotted onto the nitrocellulose and allowed to dry. The filter paper was removed and the remainder of the wells coated with type I collagen from rat tail (Collaborative Biomedical) at a concentration of 100 µg/ml in PBS. The DRG-seeded Petri dishes were maintained in explant culture medium (Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 supplemented with progesterone, insulin, sodium selenite, phosphocreatine, transferrin, and sodium pyruvate) containing 15 ng/ml nerve growth factor (βNGF; R&D Systems) at 39°C for 72 h prior to fixation in 4% paraformaldehyde. Neurite growth was monitored by phase-contrast and fluorescent microscopy and measured on captured images as described previously (3,31). A minimum of 20 DRG explants for each experimental condition were assessed and scored.

Proteoglycans for nerve culture and western blot assays

Both 'decorin' and 'biglycan' were obtained from Sigma and had been prepared from bovine articular cartilage. Certificate analyses of these products stated that protein levels had been analysed (33) in the lyophilised preparations and they complied with their specifications, which for 'decorin' was to contain ≥45% protein and for 'biglycan' to contain ≥30% protein. In addition it reported that GAG content, as determined by dimethylmethylene blue (DMB), was a minimum of 55% GAG for the 'decorin' and 70% for the 'biglycan' with uronic acid (as detected by carbazole) comprising between 20-27% of this GAG content for the 'decorin' and 14% for 'biglycan'. Following enzymatic treatment with chondroitinase ABC the major band reported on the Certificates of Analysis for both 'decorin' and 'biglycan' was reported to be a 45 kDa protein band following separation on a 10-15% SDS-PAGE electrophoresis gel, as detected by Coomassie Blue staining.

To investigate whether the GAG chains present on the SLRPs inhibit nerve growth, different enzymes were used to selectively remove specific GAG chains from 'decorin' and 'biglycan'. Chondroitinase AC (Seikagaku Corporation, Japan), which was used at concentrations ranging from 2-250 mU/ml, cleaves unsulphated chondroitin, chondroitin-4-sulphate and chondroitin-6-sulphate from the core protein and chondroitinase ABC (also used over a range of 2-250 mU/ml; Sigma) cleaves all of these in addition to dermatan sulphate (DS). Filter paper strips previously blotted with 500 µg/ml 'decorin' or 'biglycan' were incubated with the enzymes at 37°C for 2 h.

Aggrecan was prepared using previously described methods (21,34) and was extracted from the nucleus pulposus of a disc obtained post-mortem from a 70-year old male. Briefly, as described by Sivan *et al* (35), the disc was diced and aggrecan extracted using 10 volumes of 4 M guanidine hydrochloride (GuHCl) containing proteinase inhibitors in 50 mM Tris buffer, pH 7.4, at 4 °C for 48 h (36). Using centrifugation (10,000 rpm for 40 min at 4°C) the supernatant was dialyzed in the same buffer but without GuHCl. This fraction contained both aggrecan and its degradation products; using direct dissociative CsCl density gradient centrifugation in 4 M GuHCl (50 mM Tris buffer, pH 7.4) starting at a density of 1.5 g/ml (10 °C, 50,000 rpm for 48 h) the aggrecan monomers were separated in the highest density gradient fraction according to their buoyant density and molecular weight (37). The purified human aggrecan was dialyzed against distilled water and freeze-dried.

Western blot analysis of 'decorin', 'biglycan' and aggrecan

Western blotting was carried out as previously described (38) and repeated a minimum of three times. A pre-stained molecular weight standard (SeeBlue-2, Invitrogen) was run together with extracts of bovine nasal cartilage, and SLRPs and aggrecan isolated from human intervertebral disc, as positive controls, which produced bands in the region of expected molecular weight (data not shown).

PGs were deglycosylated prior to Western blotting. To remove chondroitin sulphate (CS) and DS chains, the commercially sourced 'decorin' and 'biglycan' and purified human aggrecan, were digested with chondroitinase ABC or chondroitinase AC (both at 250 mU/ml in Tris acetate buffer) or left undigested for 2 h at 37°C. In addition, they were digested with chondroitinase ABC (200 mU/ml) and keratanase (100 mU/ml) and keratanase II (2 mU/ml) in Tris acetate buffer to ensure removal of any CS, DS and keratan sulphate (KS) GAG chains. The enzymatically digested or undigested PGs were mixed with LDS PAGE application buffer (4x) and 500 mM dithiothreitol (10x) then heated for 30 min at 70°C, cooled and either 10 µg or 25 µg were loaded into each well and separated on 4-12% Bis-Tris gradient gels for 35 min at a constant voltage of 200V (Novex, Invitrogen) under reducing conditions.

Two protocols for transferring to the nitrocellulose membranes and visualisation were used. For **Protocol 1**, the separated PGs (which had been digested with chondroitinase AC or ABC) were transferred to nitrocellulose membranes (0.22 µm) using NuPAGE transfer buffer plus 10% methanol at a constant voltage of 30V for 1 h. The membranes were blocked for 1 hour with 5% skimmed milk/0.1% Tween 20 in 50mM Tris-HCl 0.15M NaCl pH 7.2 (TBS). Primary mouse monoclonal antibodies against GAGs (5D4, 2B6 and 3B3) at the concentrations provided in Table 1 were then applied to the membranes for 1 h. Membranes were rinsed in TBS and a secondary antibody applied (Mouse IgG Vectastain ABC Kit, Vector Laboratories) for 30 min followed by the avidin/biotin complex for an additional 30 min. Following colour development with 3,3'-diaminobenzidine (DAB), membranes were rinsed in distilled water and allowed to dry.

In **Protocol 2**, following separation via electrophoresis as described above, the commercial SLRPs (which had been digested with chondroitinase ABC, keratanase and keratanase II) were transferred to nitrocellulose membranes using an iBlot system (Program 3) and associated transfer stacks (all Invitrogen). These membranes were then probed for decorin, biglycan, aggrecan, fibromodulin and lumican and KS using the previously described primary antibodies (Table 1). The primary antibodies were applied using iBlot detection stacks and appropriate chromogenic kits (all Invitrogen) followed by the appropriate secondary antibody (anti-rabbit at 1:2,000 or anti mouse at 1:5,000). Membranes were rinsed in wash solution (Invitrogen) followed by autoclaved distilled water and the BCIP/NBT chromogenic substrate applied and colour development allowed for a maximum of 1 h.

Protein identification by mass spectrometry

Two different batches of the commercially sourced 'decorin' and one batch of 'biglycan' were enzymatically digested for removal of CS, DS and KS as described for Western blotting, before being separated on a 12.5% SDS-PAGE gel for mass spectrometry. Bovine nasal cartilage was treated similarly and used as a positive control. Following Coomassie Blue staining of the 12.5% gel, protein slices, spanning the entirety of each lane were excised and digested overnight at 37°C with trypsin (porcine, sequencing grade, Promega), according to the method described by Shevchenko *et al.* (39). Briefly, gel slices were washed with 0.1M NH₄HCO₃/acetonitrile (1:1), reduced with 10mM dithiothreitol in 0.1M NH₄HCO₃ at 56°C for 45 minutes followed by alkylation of cysteine residues by incubation with 55mM iodoacetamide in 0.1M NH₄HCO₃ for 30 minutes at room temperature. Prior to mass spectrometry analysis, the protein digests were cleaned up and concentrated using C18 Ziptips according to the manufacturer's recommendations (Millipore). Peptides were eluted from the Ziptips onto a stainless steel MALDI target plate with alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix at 3mg/ml (in 70% acetonitrile and 0.1% 2,2,2-Trifluoroacetic acid (TFA)) and allowed to dry. Close external peptide standards were used to calibrate the instrument in both MS and MS/MS mode. Both MS and MS/MS analysis of the peptides was performed using a 4800 MALDI TOF/TOF (AB Sciex). The mass spectrometer was operated under the control of 4000 Series Explorer software (V3.5.2 Applied

Biosystems). Peak lists of MS and MS/MS spectra were also generated with the 4000 Series Explorer software and the following parameters were used after selective labelling of monoisotopic mass peaks: MS peak lists: S/N threshold 3, Savitsky-Golay smoothing with 3 points across peak (FWHM), no baseline correction, MS/MS peak lists: S/N threshold 10, Savitzky-Golay with 7 points across peak (FWHM) and no baseline correction. Automated database searches were run using GPS Explorer software (V3.6, AB Sciex). Using MASCOT as the search engine, the Swissprot database was explored using the following parameters: precursor ion mass tolerance of 0.15Da, fragment ion mass tolerance of 0.15Da, mammalian taxonomy and finally, carbamidomethyl modification of cysteines and oxidation of methionine residues were allowed as variable modifications.

Statistical analysis

The non-parametric statistical test, Kruskal-Wallis with post-hoc analysis (Dunn's multiple comparisons test), was used to determine any differences in neurite growth on different substrata. Statistical significance was considered at $p < 0.05$.

RESULTS

Effect of 'decorin' and 'biglycan' on DRG neurite growth

Substrate choice experiments showed that 'decorin' inhibited DRG neurite growth in a dose-dependent manner (Figure 1A). Significant decreases in the average number of neurite crossings per DRG were observed between 'decorin' concentrations of 10 and 500 $\mu\text{g/ml}$ ($p=0.0012$) and 10 and 1000 $\mu\text{g/ml}$ ($p<0.0001$). No significant difference on DRG neurite growth was observed between 'decorin' concentrations of 500 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$.

Since maximum inhibition of DRG neurite growth was observed using 'decorin' at 500 $\mu\text{g/ml}$, experiments were repeated using this concentration to determine the effect of enzymatic removal of GAG chains from the 'decorin' on neurite growth. Figure 1B shows that chondroitinase ABC (which cleaves both CS and DS GAGs) at 250 mU/ml significantly reduced the inhibitory effect of 'decorin' on DRG neurite growth compared with no enzymatic treatment ($p=0.0013$). In contrast, treatment with chondroitinase AC (which cleaves CS GAGs only) did not reverse the inhibitory effects of 'decorin' on DRG neurite growth (Figure 1B).

The experiments were repeated using 'biglycan' instead of 'decorin' as the test substrate and a similar dose-dependent pattern of DRG neurite growth inhibition was observed (Figure 2A). That is, there was a significant decrease in neurite crossings when the 'biglycan' concentration was increased from 2 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$ ($p=0.0114$) and when it was increased from 2 $\mu\text{g/ml}$ to 500 $\mu\text{g/ml}$ ($p=0.0007$). No significant difference on DRG neurite growth was observed between 'biglycan' concentrations of 100 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$. Again, the effect on DRG neurite growth of the CS and DS GAG chain components of the 'biglycan' preparation were assessed by using 'biglycan' at 500 $\mu\text{g/ml}$ (as previously used to assess 'decorin') followed by either chondroitinase ABC or chondroitinase AC (both at 250 mU/ml). As can be seen in Figure 2B, inhibition of DRG neurite growth was not reversed following removal of CS or CS and DS GAG chains and the inhibitory effect of the 'biglycan' molecule was maintained.

Western blot analysis of the glycosaminoglycan chains of the commercially sourced 'decorin' and 'biglycan'

Western blot analysis of the 'decorin', 'biglycan' and aggrecan revealed the presence of KS, in all three PGs with clear 5D4-positivity (Figure 3A). Whilst the presence of KS was expected within the aggrecan preparation, it was not in the two SLRP preparations as decorin is reported to contain only 1 GAG chain of CS/DS and biglycan 2 chains of CS/DS (40). The bands for samples that had undergone enzymatic pre-treatment with either chondroitinase ABC or AC appeared to be more intense (Figure 2A; Lanes 3-4, 6-7 and 9-10) indicating that removal of the CS and DS GAG chains exposed the KS containing sugars further. The bands associated with aggrecan ran from above the 260 kDa molecular

marker down to approximately 140 kDa. The KS positive PGs in 'biglycan' and 'decorin' ran from just above the 260 kDa standard, down to approximately 60 kDa (Figure 3A).

When the membrane was probed for 4-sulphated CS using the antibody 2B6 (after chondroitinase ABC pre-treatment), additional bands were seen at approximately 45 kDa in both 'biglycan' and 'decorin' (Figure 3B). This is the expected molecular weight of the core protein of both decorin and biglycan. Since these bands were not visible in the preparations treated with chondroitinase AC, the DS GAG chains must have been more common than CS on the 'biglycan' and 'decorin' components of these commercial SLRP preparations. The membranes were also probed for the 6-sulphated CS with the antibody 3B3 and following chondroitinase ABC digestion where both aggrecan and 'biglycan' had increased immunoreactivity relating to these 6-sulphated neo-epitopes at high molecular weights (>260 kDa) whereas as expected 'decorin' did not (Figure 3C).

Mass spectrometry characterisation of commercially sourced 'decorin' and 'biglycan'

Mass spectrometric analysis of gel slices excised from sample lanes containing the two batches of 'decorin' preparations (Figure 4) resulted in the identification of several proteins with statistical significance (summarised in Table 2 and in full in Supplementary Table 1). Biglycan was the most easily detectable protein in the majority of the 'decorin' gel slices and fibromodulin and aggrecan were also detectable in several slices from each of the two preparations (Supplementary Table 1a and c). Surprisingly, decorin itself was only detected with significance in one out of eight gel slices from 'decorin' (2nd batch), while the decorin peptides detected in the 'decorin' (1st batch) preparation did not meet the criteria required for a statistically significant protein identification (see "slice 4", Supplementary Table 1b). Whilst it is not possible to derive accurate measures of relative protein quantity using this approach, it seems fair to assume that decorin is a minor component of both commercial batches of 'decorin' preparations, since it was detected with far fewer peptides than the other proteins that were detected in each sample.

Similar to the 'decorin' (2nd batch) preparation, mass spectrometry analysis of the gel slices excised from the 'biglycan' sample lane resulted in the identification of significant peptides matching to biglycan, fibromodulin, aggrecan and decorin (Table 2 and Supplementary Table 1). Though some degree of amino sequence similarity (69%) and identity (52%) exists between bovine biglycan and decorin, an alignment of the two sequences confirmed that the peptides identified in each case were not common to both proteins (Figure 5).

Validation by Western blot of the mass spectrometry data for the commercially sourced 'decorin' and 'biglycan'

Subsequent to the unexpected mass spectrometry findings, further Western blot analyses were carried out on the proteoglycan preparations. Membranes were probed for biglycan and decorin and although 'biglycan' contained biglycan and 'decorin' contained decorin, the presence of biglycan in 'decorin' and decorin in 'biglycan' (Figure 6A & B respectively) was confirmed and validated the findings of the mass spectrometric analysis (Table 2). Although the most intense immunoreactivity for the 'decorin' and 'biglycan' protein cores was observed at the expected molecular weight of approximately 45 kDa, higher molecular weight bands were also detectable for these proteins (Figure 6A & B). One of the other main findings from mass spectrometry was the presence of aggrecan within both SLRP preparations, and by probing with 6B4 (a monoclonal antibody to the amino acid sequence 413-424 of human aggrecan) its presence was also confirmed by Western blot (Figure 6C).

Further Western blot analysis of 'biglycan', both with and without chondroitinase ABC pre-treatment and undigested purified aggrecan as a positive control, clearly showed the presence of fibromodulin in 'biglycan' but none in the aggrecan preparation (Figure 6D). Additionally, the presence of low-sulphated KS (as shown with the 1B4 antibody) was detected in both 'biglycan' and aggrecan with its presence being detected at molecular weights as low as 40 - 50 kDa in 'biglycan' whilst in aggrecan, as expected, it was only detected at molecular weights of 100 kDa and greater. As a final validation step, membranes were probed for lumican; a SLRP that was not detected via mass spectrometry in 'biglycan'

and that should not be present in the purified aggrecan preparation. No positive immunoreactivity for lumican in either 'biglycan' or aggrecan was detected (Figure 6F).

DISCUSSION

Molecules that contain GAG chains have previously been shown to influence neuronal growth with those containing CS in particular being implicated in neurite inhibition (8). The aim of this study was to establish the effect on neurite growth of the two Class 1 SLRP family members, the CS/DS-containing SLRPs, decorin and biglycan. Initial findings did corroborate with those previously established with aggrecan (3,31), in that both commercially sourced 'decorin' and 'biglycan', without any enzymatic treatment, inhibited DRG neurite growth. To ensure that the observed inhibition was due to the presence of these CS/DS GAG chains, 'decorin' and 'biglycan' were enzymatically deglycosylated. For decorin this resulted, as expected, in a reduction of its inhibitory effect on neurite growth. In contrast, this effect was not observed with 'biglycan'. This triggered the further investigations we have described, using analytical techniques to decipher this anomaly.

Western blot analysis of 'decorin', 'biglycan' and aggrecan with varying degrees of glycosylation revealed the presence of KS chains on all three PG preparations. Although KS was expected to be present within the aggrecan molecule, it should not have been present in either of the SLRP preparations. In all three PG analyses, the Western blot bands appeared diffuse (Figure 3A), reflecting a variety of molecular weight moieties containing KS. Following digestion with chondroitinase ABC or AC, the intensity of the Western blot bands increased indicating that CS and DS GAGs had been present with their removal exposing additional KS epitopes which reacted with the KS-specific 5D4 antibody. Biglycan and decorin core proteins (at a molecular weight of ~45 kDa) were only visible on the membranes following chondroitinase ABC digestion (Figure 3B), implying that removal of DS GAG chains (but not CS chains) exposed these core proteins. Interestingly, following chondroitinase ABC digestion, the 'biglycan' preparation appeared to have increased immunoreactivity for the 6-sulphated 3B3 neo-epitope (Figure 3C, Arrows) at high molecular weights similarly to that seen with aggrecan. From these results, it appears that the 'biglycan' may contain a higher proportion of high molecular weight molecules, such as aggrecan, with GAG chains containing more C-6-S than the 'decorin' preparation. This, in addition to the greater amounts of high molecular weight KS containing PGs in 'biglycan', may explain the difference observed between 'biglycan' and 'decorin' when treated enzymatically to overcome neurite inhibition. These findings may also provide an explanation for the lower than expected presence of uronic acid in 'decorin' (20-27%) and even lower presence in 'biglycan' (14%), as detailed in their relevant product specifications. Typically, the CS and DS GAG chains of decorin and biglycan would contain 50% uronic acid (41).

The combination of mass spectrometry and Western blotting used in this study has demonstrated that commercial preparations of 'biglycan' and 'decorin' bought and used in studies in our laboratory actually contained many contaminating substances, at least in these specific batches. The molecules we concentrated on validating were other PG family members identified via mass spectrometry. Hence the 'decorin' also contained biglycan, fibromodulin and aggrecan, whilst the 'biglycan' also contained fibromodulin, aggrecan and decorin, at least. This renders these preparations useless for the original purpose of our study which was to unravel the contributions that different members of the PG family, known to occur in the human intervertebral disc, may make to controlling neurite extension and nerve growth.

The impact of our findings may be huge, not in the manner we originally intended but, rather in the amount of time, money and samples which have been wasted. Most importantly, however, it may impact on the possibility of erroneous information entering the scientific arena. Concern about the purity and quality of commercial chemicals and biochemicals has previously been expressed by other researchers. For example, D'Hondt *et al* (42) highlighted the impact that contaminants may have in peptide medicines, not only with respect to their presence within the approved and clinically used products, but also during the initial drug discovery phase. During this initial phase, the presence of contaminants may greatly influence functionality studies thus resulting in conclusions that are incorrect.

We know of publications which draw important mechanistic conclusions on the influence of decorin on the control of sensory neuron growth, wound-healing in glaucoma, prevention of the development of vitreoretinopathy and susceptibility to malaria. All appeared to use the same source of 'decorin' as we did. If their decorin was as impure as ours, are their results still valid? Do scientists have to check the purity of every reagent? Some researchers do prepare and validate the purity of their own preparations (43). Unfortunately most research scientists do not have the breadth of experience, knowledge or facilities to undertake this and so obtain such materials as we did, from a commercial source in good faith.

There are many sophisticated analytical techniques in the science world and it is hoped that companies will use these to provide more accurate details of the purity and constituents of their reagents and chemicals. Inherently there will be a cost to the consumer, but on balance, the reduction in time and funds spent on investigating the effects of 'impure' molecules within the scientific community, possibly resulting in inaccurate conclusions and 'flawed' studies, will offset this cost. Without this, the effect of contaminants within reagents may alter or confound the observed effect of the molecule being investigated; potentially leading to erroneous interpretation of data such as in the elucidation of mechanisms of action or the contribution a particular molecule may play in key biological processes.

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Conflict of interest: The authors declare that they have no conflicts of interest associated with this work.

Author contributions: SJB, HRF and PJ carried out the experiments and were advised by BC and SR. Data analysis was carried out by SJB, HRF, PJ, BC, SLS, CHB and SR. SJB and HRF wrote the manuscript which was edited by BC and SR.

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FIGURE LEGENDS

FIGURE 1. Graphs showing the mean \pm SD of number of neurite crossings per DRG when cultured on A) ‘decorin’ at a range of concentrations (10 – 1000 μ g/ml) without any enzymatic treatment; B) ‘decorin’ at 500 μ g/ml following chondroitinase ABC and chondroitinase AC treatment (both at 250 mU/ml) or in buffer. ** p <0.005; *** p <0.0001

FIGURE 2. Graphs showing the mean \pm SD of number of neurite crossings per DRG when cultured on A) ‘biglycan’ at a range of concentrations (2 – 500 μ g/ml) without any enzymatic treatment; B) ‘biglycan’ at 500 μ g/ml following either chondroitinase ABC or chondroitinase AC digestion (both at 250 mU/ml) or in buffer. * p <0.05; ** p <0.005

FIGURE 3. Western blot images of aliquots of purified aggrecan isolated from a human intervertebral disc (lanes 2-4), and the commercially sourced ‘biglycan’ (lanes 5-7) and ‘decorin’ (lanes 8-10) following separation on a 4-12% Bis-Tris gel. PGs in lanes 2, 5 and 8 were undigested whereas those in lanes 3, 6 and 9 and 4, 7 and 10 had been digested with chondroitinase ABC and chondroitinase AC, respectively (both 250 mU/ml). Membranes were probed sequentially with monoclonal antibodies to A) over-sulphated KS heptasaccharides (5D4); B) 4-sulphated unsaturated disaccharide CS neo-epitopes (2B6) and C) 6-sulphated unsaturated disaccharide CS neo-epitopes (3B3). Lanes 1 and 12 contain molecular weight standards. The expected molecular weight of the intact core protein of both biglycan and decorin is approximately 45 kDa (*). Aggrecan bands were particularly prominent in lane 3 (4C) following chondroitinase ABC treatment (arrow), similar bands were seen in the ‘biglycan’ preparation (Lane 6, arrow).

FIGURE 4. Image of one of the 12.5% SDS-PAGE gels stained with Coomassie Blue following electrophoretic separation of the commercially sourced ‘decorin’ (1st batches (1) and 2nd batch (2)) and ‘biglycan’ after enzymatic digestion with chondroitinase ABC, keratanase and keratanase II (removing all CS, DS and KS GAG chains). Protein slices as outlined (1–8) were dissected from each lane then prepared for mass spectrometry analyses.

FIGURE 5. Sequence alignments highlighting biglycan peptides identified by mass spectrometry analysis. The ClustalW2 sequence alignment tool was used to align the amino acid sequence for bovine decorin (P21793) and bovine biglycan (P21809). A) Biglycan peptides that were identified in both ‘decorin’ samples are shown in red. Additional peptides identified only in the ‘decorin’ (2nd batch) preparation are shown in blue. B) Decorin peptides that were identified in the ‘biglycan’ sample are shown in red.

FIGURE 6. Western blot images of aliquots of the commercially sourced ‘decorin’ and ‘biglycan’ and purified human aggrecan following separation on 4-12% Bis-Tris gels. The PGs in A-C had been digested with chondroitinase ABC, keratanase and keratanase II (removing CS, DS and KS GAG chains). Lanes 1, and 3 of A-C contain different batches of ‘decorin’, lane 4 contains ‘biglycan’ and lane 2 the molecular weight standards. The expected molecular weight of the intact core protein of both biglycan and decorin is approximately 45 kDa (*). The membranes were probed with antibodies to A) biglycan (PR-85); B) decorin (DS-1) and C) aggrecan (6B4). For the membranes in D-F, lane 1 contains the molecular weight standards; lanes 2 and 3 contain ‘biglycan’ without and with prior enzymatic digestion with chondroitinase ABC, respectively and in lane 4 is purified human aggrecan without prior digestion. These membranes were probed with D) fibromodulin; E) 1B4 which detects less-sulphated KS and F) lumican.

TABLE 2. A summary of the proteins that were identified with significance in ‘biglycan’ and ‘decorin’ preparations by mass spectrometry analysis. Only proteins that were identified with statistical significance (i.e. $p < 0.05$) are shown. The number of MS peptides refers to the number of peptides that were matched to each protein of bovine origin and the number of MS/MS peptides refers to the number of peptides for which it was possible to generate matching ions.

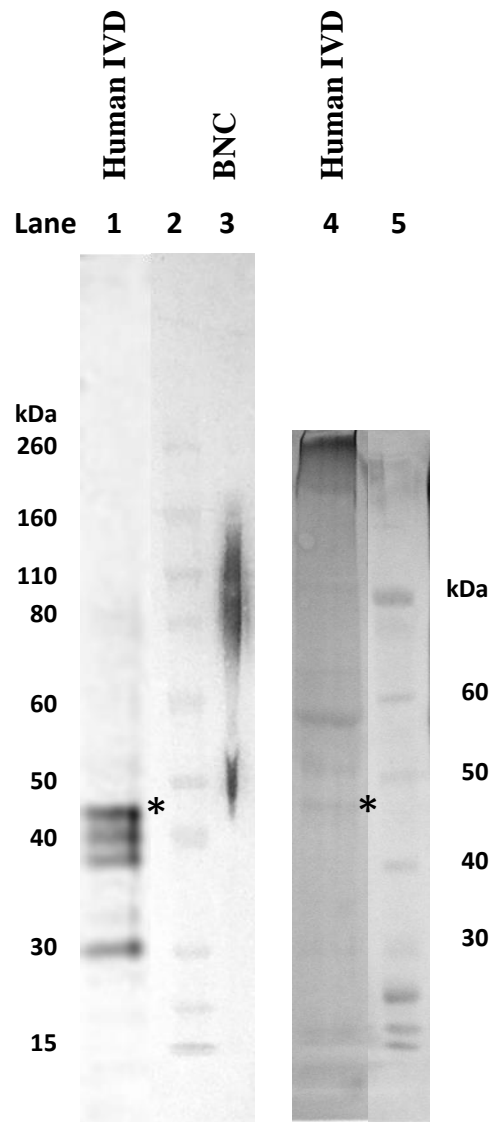
Sample	Proteins detected with significance*	Number of MS peptides	Number of MS/MS peptides
‘Decorin’ (1 st batch)	Biglycan	14	6
	Fibromodulin	8	4
	Aggrecan	14	4
‘Decorin’ (2 nd batch)	Biglycan	15	8
	Fibromodulin	9	5
	Aggrecan	18	5
	Decorin	5	2
‘Biglycan’	Biglycan	16	7
	Fibromodulin	8	4
	Aggrecan	23	8
	Decorin	9	3

*Due to inherent issues with database redundancy, significant protein matches are often made to multiple species (because of sequence homology). In this instance, because we know the preparations were of bovine origin, only significant hits that were assigned to this species are summarised here. For full protein and peptide summaries of each gel slice please refer to Supplementary Table 1.

TABLE 1. Summary of the antibodies used during Western blotting to identify the presence of decorin, biglycan, aggrecan, fibromodulin and lumican core proteins and keratan sulphate (KS) glycosaminoglycan chains and chondroitin sulphate (CS) neo-epitopes ('stubs') following enzymatic treatment.

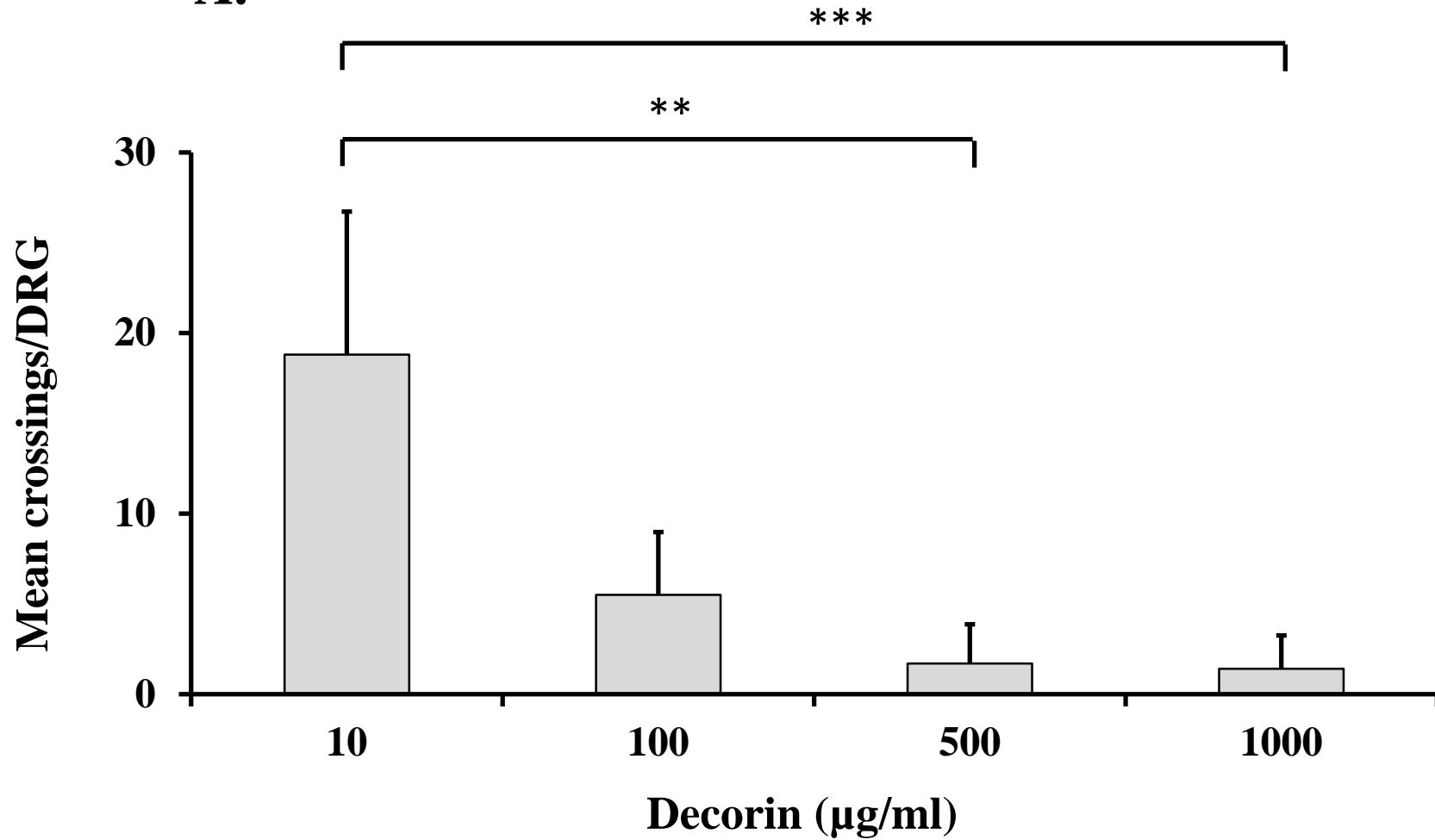
Antibody	Dilution	Detects	Epitope	Ref
mAb DS-1	1:125	Decorin	C-terminal of core protein	(44)
pAb PR-85	1:500	Biglycan	C-terminus (CGG)TDRLAIQFGNYKK	(45)
mAb 6B4	1:50	Aggrecan	Amino acids 413-424 of human aggrecan	(46)
pAb PR-184	1:500	Fibromodulin	C-terminus (CGG)LRLASLIEI	(45)
pAb PR-586	1:500	Lumican	C-terminus (CGG)LRVANEITVN	(47)
mAb 5D4	1:500	KS	Over-sulphated KS heptasaccharides	(48)
mAb 1B4	1:2500	KS	Low-sulphated KS	(49)
mAb 2B6	1:50	C-4-S 'stubs'	4-sulphated unsaturated disaccharide CS neo-epitopes	(50)
mAb 3B3	1:50	C-6-S 'stubs'	6-sulphated unsaturated disaccharides CS neo-epitopes	(51)

All antibodies were provided by B Caterson except for those against biglycan, fibromodulin and lumican which were kindly provided by Peter Roughley, and to decorin (purchased from Developmental Studies Hybridoma Bank, Iowa).

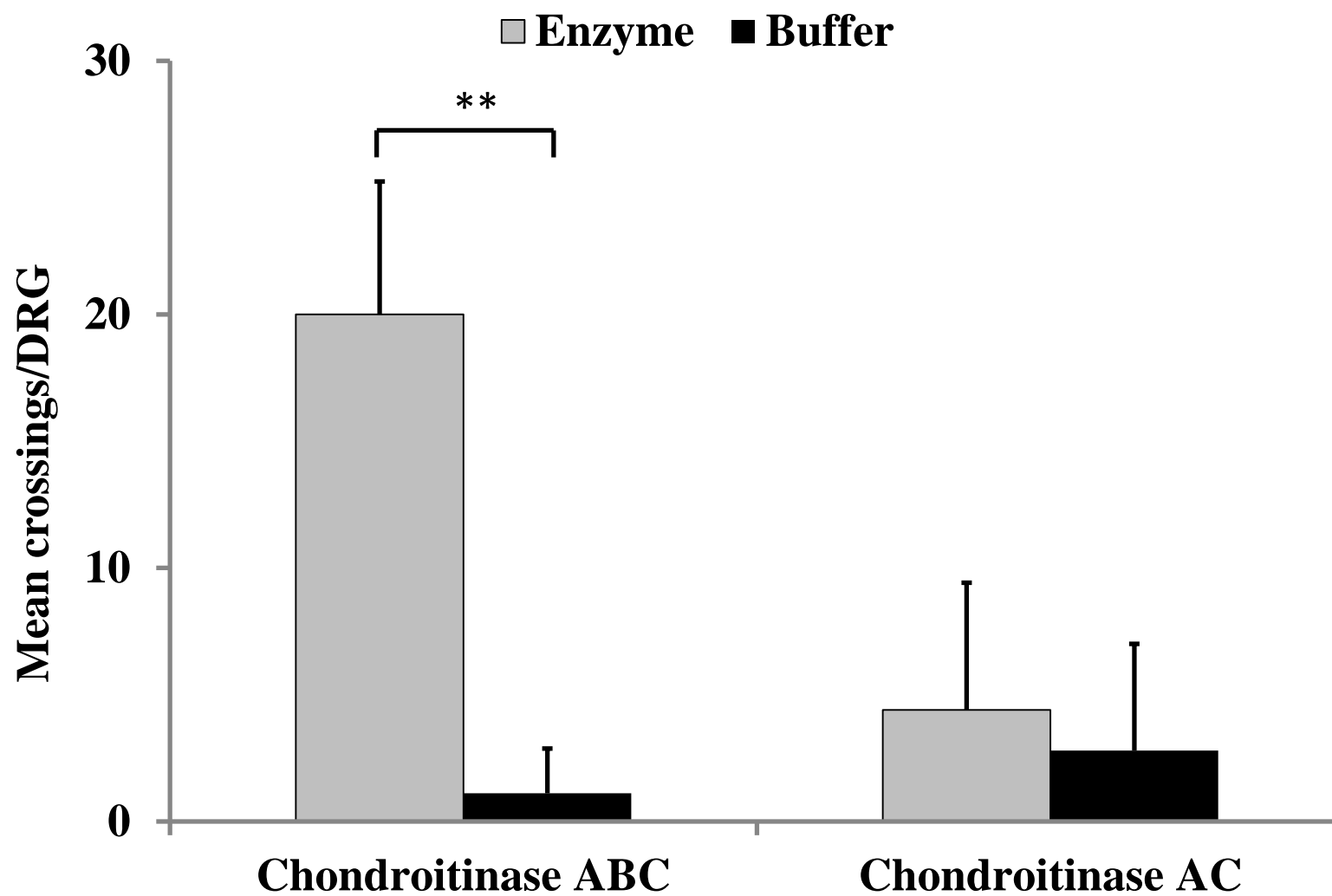


ADDITIONAL FIGURE. Western blot images of positive controls for biglycan and decorin following separation on 4-12% Bis-Tris gels. Lanes 1 and 4 contain small leucine-rich proteoglycans isolated from human intervertebral disc (IVD) which have been enzymatically digested with chondroitinase ABC and keratanase to remove CS, DS and KS GAG chains, as previously described by Brown *et al.* (23). Lane 1 was probed for biglycan (PR-85) and lane 4 for decorin (DS-1). Lane 3 contains an extract from bovine nasal cartilage (BNC) without prior enzymatic digestion (GAG chains still present) which has been probed for decorin (DS-1). Lanes 2 and 5 contain molecular weight standards. The expected molecular weight of the intact core protein of both biglycan and decorin is approximately 45 kDa (*).

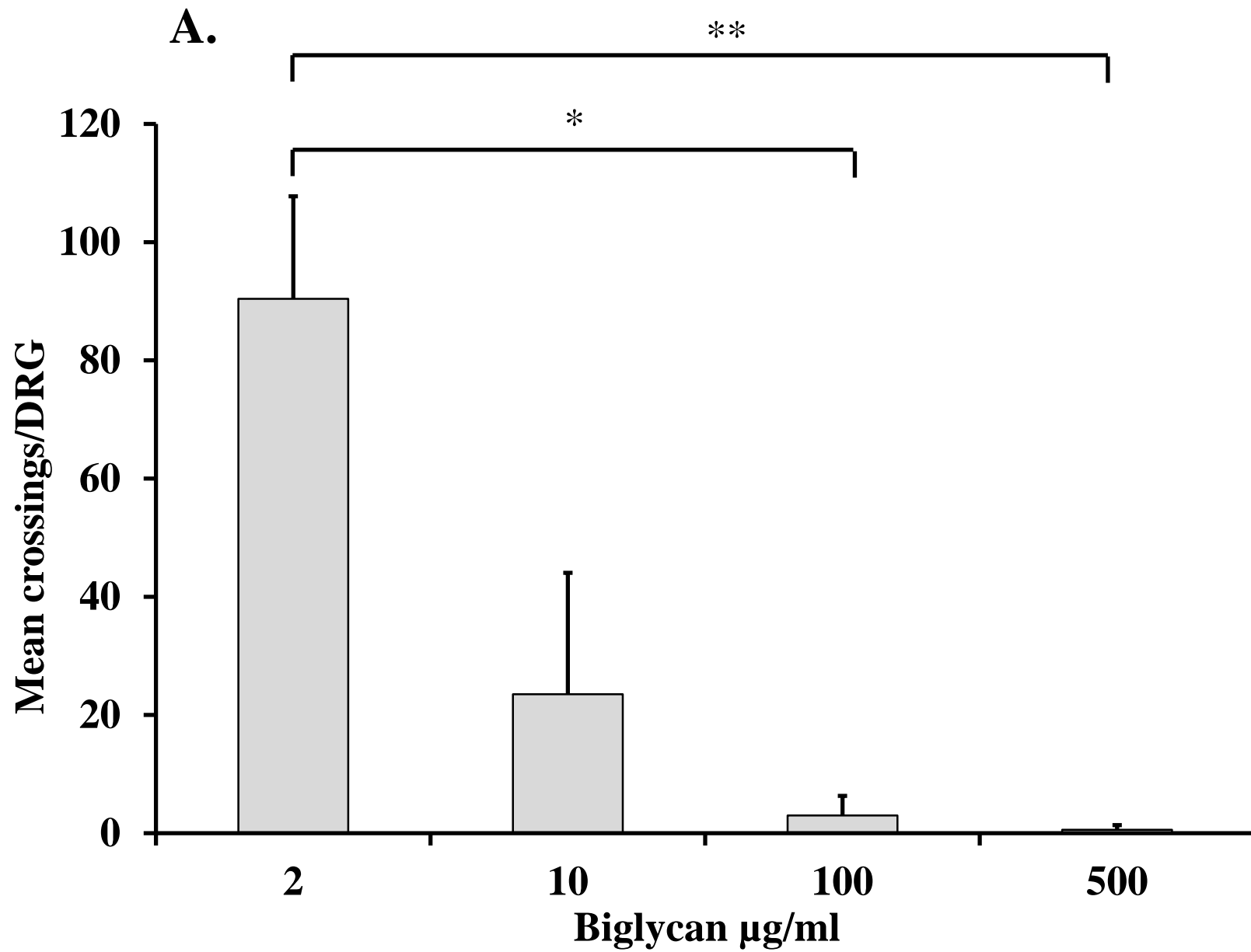
A.



B.

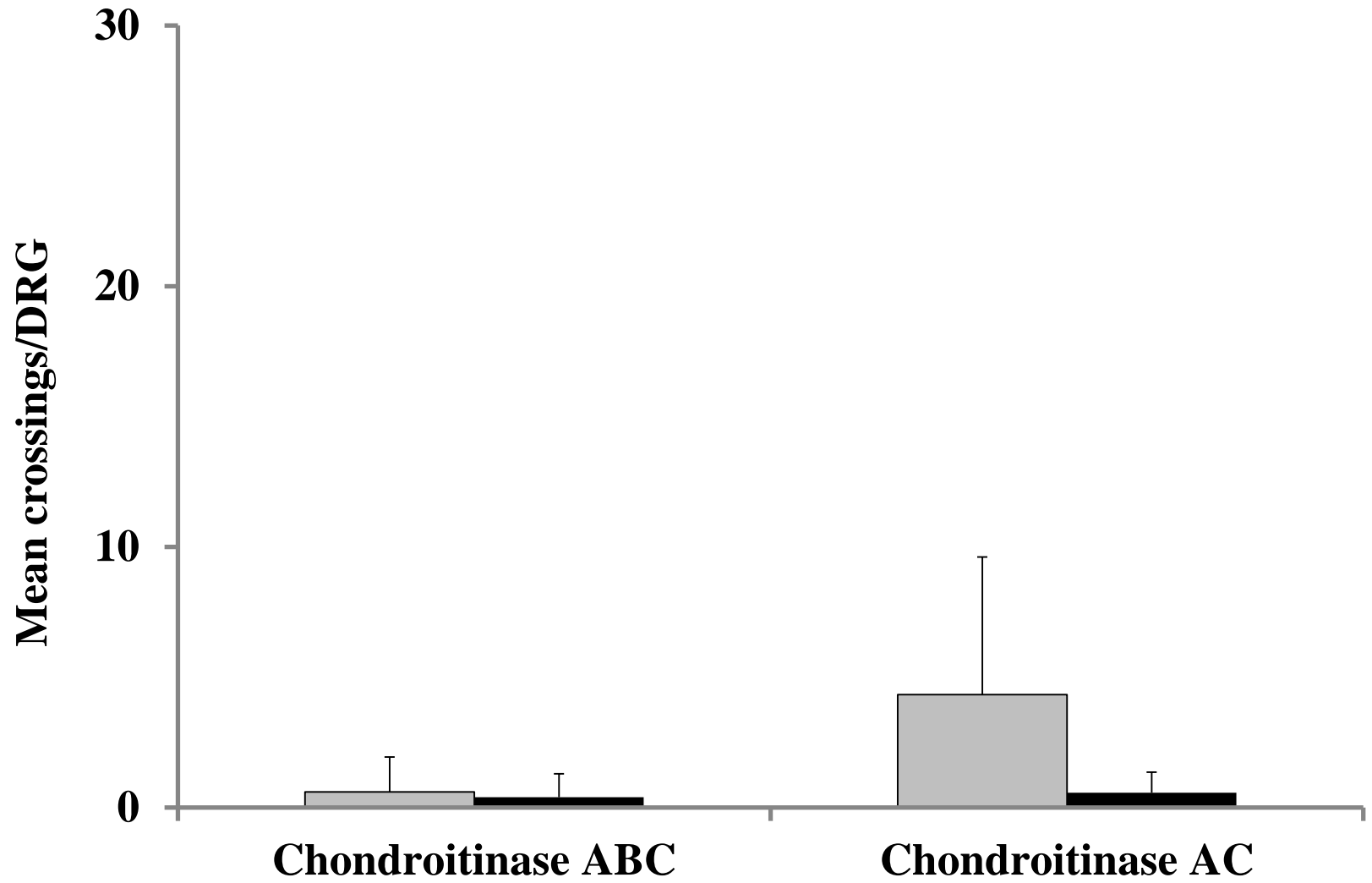


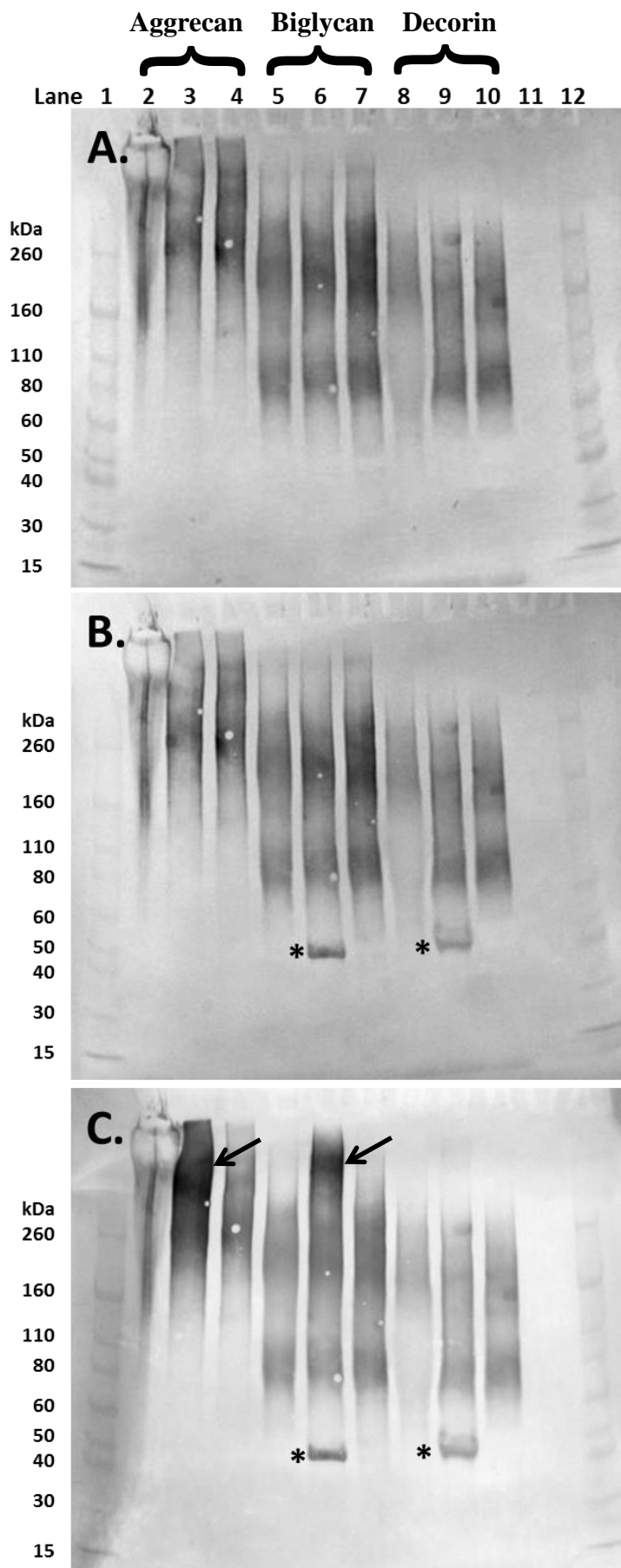
A.

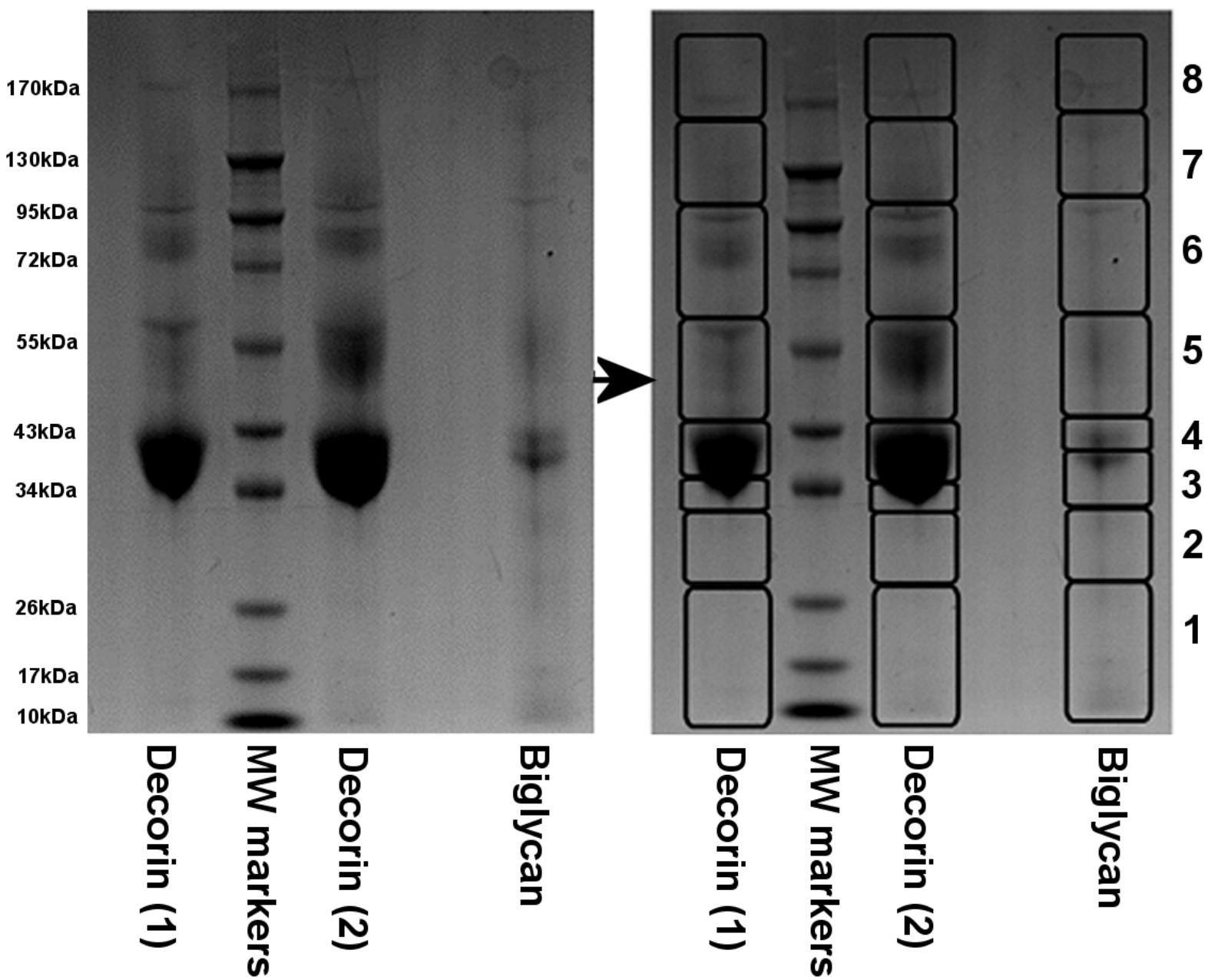


B.

■ Enzyme ■ Buffer







A

Decorin (bovine)	MKATIIIFLLVAQVSWAGPFQOKGLDFDMLD-----EASGIGPEEHFPEVPEIEP-M	51
Biglycan (bovine)	MWPLWPLAALLALSQALPFEQKAFWDFTLDDGLPMLNDEEASGAETTSIGIPDLDSLPTTY	60
	* . : : * * * : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	GPVCPFRQCCHLRVVQCSLDGLEKVPKDLPPDTALLDLQNNKITEIKDGFKNLKNLHTL	111
Biglycan (bovine)	SAMCPFGCHCHLRVVQCSLDGLKAVPKEISPDITLLDLQNNDISSELRKDDFKGLQHLAL	120
	.. : * * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	ILINNKISKISPGAFAPLVKLERLYLSKNQLKELPEKMPKTLQELRVHENEITKVRKSVF	171
Biglycan (bovine)	VLVNNKISKIHEKAFSPRLKQKLYISKNLHVEIPPNNLPSSLVELRIHDNRIRKVPKGVF	180
	: * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	NGLNQMIIVVELGTNPLKSSGIENGAFQGMKKLSYIRIADTNITTIPQGLPPSLTELHLDG	231
Biglycan (bovine)	SGLRNMNCIEMGGNPLENSGFEPGAFDGLK-LNYLRISEAKLTGIPKDLPETLNEHLHDH	239
	. * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	NKITKVDAASLKGLNNLAKLGLSFNSISAVDNGSLANTPHLRELHLNKNLKVKVPGLAD	291
Biglycan (bovine)	NKIQAIELEDLLRYSKLYRLGLGHNQIRMIENGSLSFPLTLRELHLDNNKLSRVPAGLPD	299
	*** : : . * . : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	HKYIQVVYLHNNNISAIGSNDFCPPGYNTKKASYSGVSLFSNPVQYWEIQPSTFRVCVYVR	351
Biglycan (bovine)	LKLLQVVYLHTNNITKVGNDFCVGFVGVKRAYNGISLFNNPVPYWEVQPATFRVCVTD	359
	* : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	AAVQLGNYK- 360	
Biglycan (bovine)	LAIQFGNYKK 369	
	* : * : * : *	

B

Decorin (bovine)	MKATIIIFLLVAQVSWAGPFQOKGLDFDMLD-----EASGIGPEEHFPEVPEIEP-M	51
Biglycan (bovine)	MWPLWPLAALLALSQALPFEQKAFWDFTLDDGLPMLNDEEASGAETTSIGIPDLDSLPTTY	60
	* . : : * * * : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	GPVCPFRQCCHLRVVQCSLDGLEKVPKDLPPDTALLDLQNNKITEIKDGFKNLKNLHTL	111
Biglycan (bovine)	SAMCPFGCHCHLRVVQCSLDGLKAVPKEISPDITLLDLQNNDISSELRKDDFKGLQHLAL	120
	.. : * * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	ILINNKISKISPGAFAPLVKLERLYLSKNQLKELPEKMPKTLQELRVHENEITKVRKSVF	171
Biglycan (bovine)	VLVNNKISKIHEKAFSPRLKQKLYISKNLHVEIPPNNLPSSLVELRIHDNRIRKVPKGVF	180
	: * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	NGLNQMIIVVELGTNPLKSSGIENGAFQGMKKLSYIRIADTNITTIPQGLPPSLTELHLDG	231
Biglycan (bovine)	SGLRNMNCIEMGGNPLENSGFEPGAFDGLK-LNYLRISEAKLTGIPKDLPETLNEHLHDH	239
	. * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	NKITKVDAASLKGLNNLAKLGLSFNSISAVDNGSLANTPHLRELHLNKNLKVKVPGLAD	291
Biglycan (bovine)	NKIQAIELEDLLRYSKLYRLGLGHNQIRMIENGSLSFPLTLRELHLDNNKLSRVPAGLPD	299
	*** : : . * . : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	HKYIQVVYLHNNNISAIGSNDFCPPGYNTKKASYSGVSLFSNPVQYWEIQPSTFRVCVYVR	351
Biglycan (bovine)	LKLLQVVYLHTNNITKVGNDFCVGFVGVKRAYNGISLFNNPVPYWEVQPATFRVCVTD	359
	* : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
Decorin (bovine)	AAVQLGNYK- 360	
Biglycan (bovine)	LAIQFGNYKK 369	
	* : * : * : *	

